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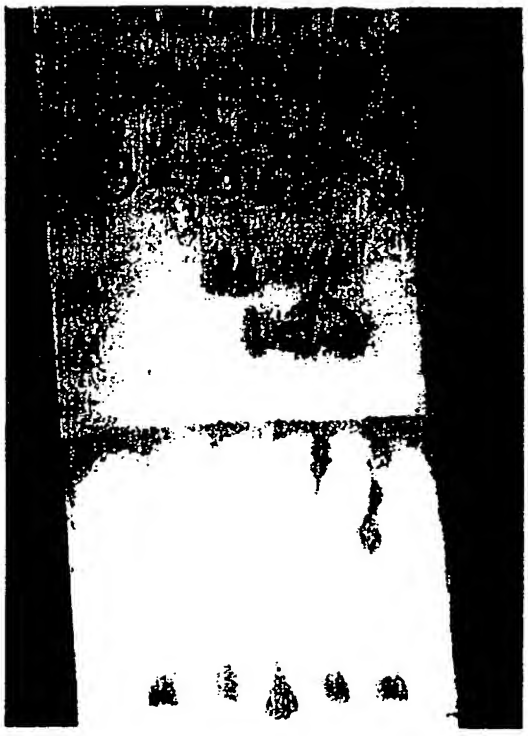

(57) Abstract: Methods are described for rapid profiling of characteristics of chemical compounds that are useful indicators of the potential efficacy of individual chemical compounds for a particular intended use and/or desired route of administration, as well as information relative to potential chemical and physical stability. The methods use small (<10 mg) amounts of a chemical compound to obtain a complete profile of characteristics. A database generated from the profiles can be searched to identify chemical compounds having a desired set of characteristics.

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HIGH THROUGHPUT CHEMICAL PROFILING

INTRODUCTION

Field of the Invention

The present invention is concerned with massively parallel testing of chemicals to develop a profile of potential lead compounds as a way of identifying and characterizing bioactive leads for the development of pharmaceuticals, agrochemicals, and the like.

Background

Drug development processes are lengthy: drug discovery, preclinical testing, human clinical trials and FDA approval constitute the majority of time expended in the process of new drug development. A conventional process involves testing and screening thousands of individual compounds for a desired therapeutic activity. Historically, less than 1 in 10,000 potential compounds have successfully made it to the drug market, at costs of greater than $200 million per drug; it typically requires greater than 10 years from discovery to approval.

With the development of combinatorial chemistry, there has been a drug discovery paradigm shift; in the past, limited numbers of compounds were available and screening *in vitro* and *in vivo* was labor and cost intensive. With the development of new screening processes which have the potential for high throughput screening, many compounds per unit time can be tested individually *in vitro* for a particular biological activity. Such screening techniques generally involve testing for inhibitors of specific protein functions, or binding to particular cellular receptors or other targets, but do not provide information on other factors which may affect the ultimate suitability of a test compound for its intended purpose as, for example, a pharmaceutical product. Indeed, it is often not until the stage of expensive late preclinical testing or clinical trials is reached that promising candidate drugs fail due to any of a variety of reasons, including undesirable biological effects, low bioavailability, poor results in clinical trials, poor drug pharmaceutical properties, and poor results in animal studies; in other words, the overwhelming reason for new drug failure is the inability to predict the effects of new drugs on biological test subjects in preclinical models. The result is that

researchers must stop further development on a failed drug and initiate new research for discovery and the development of better leads. Massive numbers of compounds are being developed using combinatorial chemistry. Many "hits" are identified from high throughput screening (HTS) resulting in a bottleneck shift: sorting out false positives from among the hits to identify true "winning" leads as new drug candidates. The problem is that false positive drug hits are common and require significant expense to uncover: most hits/leads fail after a large investment of time and money. In many cases, failure is not identified until extensive chemical synthetic scale-up (gram-kilogram level) has been made and *in vivo* testing has been performed (12-24 months post discovery).

Advances in combinatorial chemistry coupled with automated ultra high throughput screening (UHTS) will be the foundation for drug discovery as we enter the next century. The accelerated pace at which new drug targets from genomics are discovered, as well as, the need for improved newer generations of drugs for established disease targets will fuel the need for high-yield cost-effective methods that will produce "true" high quality lead drug candidates. Combinatorial chemistry allows for the rapid generation of 10^3 - 10^6 compounds per target. Currently, large pharmaceutical company chemical libraries range from 0.5 - 1.0 x 10^6 compounds. Fully automated UHTS systems can evaluate 10^3 - 10^4 compounds per target per day for biological activity. A hit rate of 0.1% will yield 1-10 compounds per day that may require additional synthesis for further biological testing and/or synthesis of additional analogs for lead optimization. It is therefore of interest to develop rapid testing methods that provide a maximum amount of information from small quantities of test compounds about a potential drug candidate early in the discovery process before the investment of significant amounts of time and research dollars.

SUMMARY OF THE INVENTION

The present invention is directed to a method for high throughput profiling of biological, physical and chemical characteristics of a test agent to produce a database that can be used to predict or model for such critical development criteria as bioavailability, carcinogenicity, potential toxicities and pharmaceutical properties such as diluent/excipient interactions, general molecular interactions and general drug stabilities. The method includes the steps of contacting multiple replicates of the agent under dynamic conditions with a parallel array of effectors and

for a time sufficient for a migration pattern to develop for each agent as a result of interaction with each of the effectors as compared to one or more controls. Several parallel arrays of effectors can be used and the information combined to construct a multidimensional space of characteristics for the agent. Also included is a method for identifying agents which bind to a target molecule of interest which includes the step of contacting a parallel array of the agents with multiple replicates of the target molecule of interest. The invention finds use in providing a preview of the pharmaceutical potential of an agent, as well as, insight into the pharmacokinetic and/or pharmacodynamic properties of the agent, and for identifying agents which have particular characteristics of interest. As an example, the method can be used to screen many compounds *in vitro* with assays that help predict whether oral bioavailability is possible for a potential pharmaceutical.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a photograph of a TLC plate viewed under chartwave UV light for test described in Tables 4 and 6. The migration pattern shows that Epinephrine (Epi) as a test drug was affected by $KMnO_4$ and to a lesser extent by DNA in terms of the development of a degradant.

Figure 2 shows a photograph of a TLC plate viewed under shortwave UV light for test described in Tables 4 and 6. The migration pattern shows that Doxycycline (Doxy) was affected by an interaction with $KMnO_4$ and an with phosphatidyl choline (PC).

Figure 3 shows a photograph of a TLC plate viewed under shortwave UV light for test described in Tables 5 and 7. The migration pattern shows that Ethidium bromide (EtBr) bound to DNA, and that EtBr was sequestered by G-C, and A-T nucleic acid polymers.

BRIEF DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods are described for high throughput profiling of characteristics of an agent to provide information about the agent including its physical stability, chemical stability and biological availability and for identifying agents which bind to target molecules of interest. To obtain profiles of the characteristics of an agent, multiple replicates of small amounts of an agent are contacted simultaneously on a solid support generally under dynamic conditions with a parallel array of effectors, and any interactions evaluated, using for example TLC methods,

electrical charge, capillary action or a combination of these methods. By a parallel array of effectors is intended a multiplicity of agents in a single assay panel. By dynamic conditions is intended conditions which provide for movement of at least one of the agent, the individual effector, agent-effector complexes, and/or degradation products of the action of the effector on the agent for a time sufficient for a detectable migration pattern of at least one of these compositions to develop. By the term agent is intended a compound or a composition which is a molecule of interest, for example a candidate drug, a pesticide, an environmental contaminant, a member of a specific binding pair such as a receptor ligand, an antigen or an antibody. By the term effector is intended any molecule or condition which has the potential to alter a test agent, for example by promoting degradation of the agent, by complexing with the agent, and the like. The effectors include physical effectors, such as increased or decreased temperature, and lighting changes; chemical effectors, such as agents which alter pH, oxidizing agents; reducing agents, and metal ions; effectors which alter bioavailability, particularly biomolecules such as DNA, RNA, and proteins such as serum albumin, membrane lipids and components of biological fluids and degradative enzymes.

Where a complex is formed, the complex can be as a result of covalent binding, specific binding or non-specific binding. By specific binding is intended binding such as that between members of a specific binding pair that has a high affinity, usually, a kD of about 1×10^{-6} to 1×10^{-12}M, generally greater than about 1×10^{-9}M. By non-specific binding is intended non-covalent binding that is generally of low affinity, usually a kD of about 1×10^{-3}M to 1×10^{-6}M. Non-specific binding may result from a hydrophilic or a hydrophobic interaction between molecules.

Multiple arrays of effectors can be combined to develop a fingerprint profile of a particular agent based upon individual migration patterns obtained with the effectors. By fingerprint is intended information as to an array of individual characteristics of a particular agent which can be visualized as a multidimensional space of characteristics. To identify agents which bind to target molecules of interest such as DNA, receptor sites, substrates for particular enzymes, and the like, a parallel array of agents is contacted with multiple replicates of the target molecule of interest.

The subject invention offers several advantages over existing technology. The method provides a fundamental characterization of information required for an early understanding of a test compound's pharmaceutical or other potential. Early elimination of false positive leads is

achieved by screening to identify and rank order possible candidates and thereby eliminate false positives in a dramatically shortened time which provides an early go or no go decision on more extensive *in vivo* testing. Furthermore, enhanced quantitative structure activity relationship (QSAR) analyses typically used by medicinal and synthetic chemists to identify trends of critical parameters which correlate with biological activity can now be developed with many rather than a limited number of test parameters (C. Hansch and A. Leo "Exploring QSAR", 1995, ACS Prof. Ref. Book, American Chemical Society). This results in reduced costs by limiting scale-up and *in vivo* testing to only those compounds with an appropriate fingerprint and/or multidimensional space of characteristics. The technology also has the advantage that massively parallel *in vitro* assays can be run using automated analysis systems to provide multiparameter profiles for drug candidates which can be coupled with other chemical and bioinformatics and data management systems. Furthermore, the information can provide chemists with better direction for additional chemical syntheses for lead drug optimization.

Other advantages include that the system is high throughput. The multiparameter profiles that are developed yield increased confidence in lead drug discovery and optimization because drug candidate selection is made with substantial data support. The high failure rate and extensive time and investment of developing new drugs thus can be averted by obtaining more valuable information about a test compound's attributes and vulnerabilities during the initial drug-screening phase. This aspect is particularly important in view of the rapidly growing number of leads being developed using combinatorial chemistry; by clearing the path for "true/winning" positive leads to be evaluated, new drug development is accelerated. The methodology has the advantage that it identifies alternate potential biological targets or sources of toxicity for a given test compound and provides guidance as to the potential of more value-added routes of administration, for example, oral rather than intravenous delivery.

For the development of novel therapeutics that address large patient populations, it is highly desirable that a compound under preclinical development be orally bioavailable because oral bioavailability affords a number of important benefits. Testing for oral availability can be performed rapidly using the subject methodology with a small quantity of test drug, typically less than about 10 mg. This is in contrast to traditional testing where the determination of whether a lead compound is orally bioavailable typically requires initial testing in rodent experimental models. This determination can require that a radioactive label to be attached to the test

compound so that the fate of the molecule can be followed after oral dosing. This testing is costly, time consuming, often requires additional synthesis, and requires that the researchers make the decision that the compound chosen was a "best bet". In many cases, the amount of biological chemical and physical information about the compound is limited and in most cases there would be other compound candidates that may be also worthy of additional testing, particularly, *in vivo*. Additional advantages of the subject methodology include that the data generated from test agents can be compared to data generated from established drugs with known oral bioavailability, thus providing/identifying those effectors/conditions that are critical for predicting bioavailability. The information, whether favorable or not, provides insights to medicinal chemists as to the direction of future synthesis for lead optimization in a more timely, cost effective manner than is currently practiced.

To obtain a profile of characteristics of an agent, an assay device such as that described in copending application attorney docket No. MECO.002.00USP filed on even date herewith, can be used. The device used has at least three primary regions, a central region containing a means for separate massively parallel analysis of an interaction between a test compound and select effectors and, in fluid connection at either end, a starting reservoir and a receiving reservoir. The means preferably is multiple racetracks, such as used in the above-identified assay device. In one embodiment, the starting reservoir contains a test agent in a suitable solvent and the racetracks are each filled with different effectors, the effectors generally are provided as parallel arrays of conditions and/or compounds for assessing particular characteristics of interest relating to a test agent. Alternatively, the starting reservoir contains an effector in a suitable solvent and the racetracks are each filled with different test agents. In a different embodiment, the starting reservoir contains a suitable solvent and the racetracks each contain one or more layers of absorbent material which have been impregnated with a test agent and an effector. Either the test agent or the effector can be present in multiple replicates, depending upon the experimental design and purpose of the experiment. As a control, at least one racetrack contains test agent alone (no effector) or effector alone (no test agent), whichever is present in multiple replicates. A listing of some examples of effectors is presented in Table 1, below.

The agent can be any compound for which it is desired to obtain a profile of characteristics, including drug leads and other compounds intended as antineoplastic agents, pesticides, fungicides, antibiotics, antiviral agents and the like. Agents can include known drugs

as an aid in discovering new uses or new routes of administration, and potential drug candidates, such as drug candidates which are structurally related to known drugs, particularly those which have undesirable side effects, which lack a desired specificity, and / or which are marginally efficacious. The test agent is dissolved in a suitable solvent for a pharmaceutical, such as physiological saline, ethanol, ethylacetate, or dimethylsulfoxide; for agricultural products, diluents used for sprays, and the like can be used. The amount of test agent used per assay generally is in a concentration of about 0.001 mg/ml to 10 mg/ml, preferably about 0.01 mg/ml to 1 mg/ml, more preferably about 0.1 to 0.5 mg/ml. Reaction mixture volumes can range from about 1 to 2000 µl, more usually about 2 to1000 µl.

Unless it is an effector, moderate, and desirably substantially constant, temperatures are normally employed for carrying out the method. The temperature employed during the method is usually about 4°C to 50°C, more usually about 15°C to 40°C. For most biological applications, a temperature of about 20°C to 37°C is preferred. The ionic strength and viscosity of the medium are optimized for a particular application. The ionic strength of the medium is usually about 0.005 to 0.6 mM, more typically, about 0.02 to 0.10 mM. Isotonic conditions are preferable for most biological assay applications. The time period for carrying out the method is usually from about 0.2 seconds to 24 hours, more usually, from about 2 minutes to about 4 hours, generally about 5 minutes to 1 hour. The time period depends on the temperature and the anticipated rate of interaction of the test agent with the effector.

TABLE 1

Types of Effectors That Can Be Employed in Parallel Testing/Profile Screening Assays

Physical Effectors

Temperature Exposure

Light Cycles

1. Various Wavelengths including IT → Uv

Chemical Effectors

Estimates of pharmaceutical stability

1. Oxidative stress
   a. sodium persulfate
   b. sodium hypochlorite
   c. potassium permanganate
2. Reducing agents

a. sodium borohydride
b. mercaptoethanol

Metal Ions

1. Chelation- catalytic redox reactions
   a. Fe^{+2+3}
   b. Al^{+3}
   c. Pb^{+2}
   d. Ca^{+2}

pH effectors

1. pH ranges (pH 2-10) vs time
2. acid range
   HCl
   $NaHSO_4$
3. Alkaline range
   NaOH
   Na_2CO_3

Biological Effectors

A. Macromolecules

1. Structural Proteins
   a. albumin
   b. fibrogen / tofibin / thrombin
   c. collagens
   d. tubulin
   e. elastin
2. Extracellular matrix components
   a. hyaluronic acid
   b. heparin
   c. synovial fluids
3. Membranes & Lipids
   a. cholesterol
   b. phosphetdyl choline
   c. brain lipids
4. Nucleic acids and polymers
   a. individual nucleotides A T C G U
   b. homopolymers single strand poly A, T, G, L
   c. homopolymers double strand G-C, A-T

B. Biological Fluids

1. whole blood
2. serum
3. plasma
4. lymph
5. saliva
6. gastric fluids
7. synovial fluids
8. tears
9. bile
10. pancreatic juice

11. feces
12. urine

C. Biological Tissues / Extracts

1. Organ homogenates / superactants
    a. brain
    b. glandular tissues
    c. visceral organs
    d. skin
    e. muscle
    f. gastrointestinal tract
    g. liver
2. Adipose tissue

D. Agents Affecting Bioavailability

1. Oral Drug Delivery
    a. food stuffs
    b. carbohydrates
    c. fiber
    d. fats
    e. oil
    f. protein
2. Intravenous
    a. serum proteins
    b. red blood cells

Effectors may be prepared in a variety of ways. If stable, stock materials generally are made in a defined concentration and preapplied to the components of the solid support used in the testing device. Other effectors, if unstable long term in solution, are prepared and lyophilized, then rehydrated and applied to the testing device prior to use and exposure with the compounds of interest to profile. Each effector can be made in a reproducible, stable fashion that allows for inter experiment analysis. The test agent and effector can be mixed prior to application to the absorbent material, or can be spotted onto the absorbent material individually at the same or different locations within individual racetracks. When the test agent and the effector are on separate layers of absorbent material, the layers are overlaid so that the test agent and the effector are in contact either prior to or during induction of solvent flow from the starting reservoir through the racetracks in the direction of the receiving reservoir. Where the agent and the effectors potentially are each members of a specific binding pair, generally the test agent is present in an amount sufficient to saturate the binding site for the test agent on the effector. Preferred ratios range from 1:1 to 5-6 log excesses of the test agent or effector. The range of molar ratios of test drug to effectors may encompass 10^{-10} to 1 moles.

An array of effectors for evaluating chemical effects can include compounds to alter the pH to which the agent is exposed, including a pH range of about 2-10; in the acidic range, effectors can include acids such as hydrochloric acid, acetic acid and sulphuric acid, and in the alkaline range, effectors can include bases such as sodium hydroxide and sodium carbonate; compounds which are oxidizers, such as sodium persulfate, potassium permanganate; sodium hypochlorite, sodium perchlorate, sodium perborate, ferric ion, iodine, osmium tetroxide, and transition metals such as vanadium, chromium, manganese and the like; compounds which are reducers, for example, sodium borohydride and mercaptoethanol, sodium hydride, Raney nickel, amalgams of mercury and sodium or potassium; metal ions involved in chelations and/or catalytic redox reactions, such as ferrous and ferric ions, magnesium and aluminum ions, lead ions, and calcium ions, and transition metal ions, including platinum and palladium. The amount of the effector generally is in the range of 10^{-9} to 10^{-2} moles.

An array of effectors for evaluating potential efficacy of a test agent can include biological targets of the test agent, including macromolecules such as DNA and RNA, particularly from a potential target organism, i.e. mammalian, bacterial, etc. As an example, the biological functions of some classes of small molecules can be correlated with their interactions with nucleic acids including DNA and RNA, double stranded or single stranded in varying sizes and polymers including single stranded homopolymers (poly A,T,G, C) and double stranded homopolymers (G-C, A-T), nucleotide and individual nucleotides. Defined sequences and specific genes also can be evaluated. Depending upon the intended use of the test compound, binding to DNA can be either a positive or a negative trait. For many small ligands, an ordered, double-stranded polymer structure is a prerequisite for binding, e.g., acridines (Blake and Peacocke (1968) *Biopolymers* 61:1225-1253), actinomycin (Muller and Crothers (1968) *J. Molec. Biol.* 35:251-290) and ethidium bromide (LePecq and Paoletti (1967) *J. Molec. Biol.* 27:87-106). Such binding indicates that the test compound is intercalating between nearest neighbor bases and may be a good antineoplastic agent. Compounds that do not bind measurably to double-stranded polynucleotides are unlikely to intercalate, cross-link or alkylate.

A preference for one of the four major bases as the site of association can also be evaluated as this is not uncommon, e.g., actinomycin has a preference for guanine (Kersten (1961) *Biochem. Biophys. Acta* 47:610-611; Goldberg *et al.* (1961) *Proc. Nat'l. Acad. Sci. USA* 48:2094-2101). Actinomycin binding to DNA additionally appears to depend not only on

guanine but also on the base sequence (Wells (1969) *Science* 165:75-76). However, in general, unique base sequences are not required for the binding of small ligands. The biomolecule to be used as an effector can be a native biomolecule or the biomolecule can be denatured so as to observe whether there is an interaction with the single-stranded molecule, particularly if there is no interaction with the double-stranded molecule. As an example, steroid binding to DNA requires the denatured form (Cohen and Kidson (1969) *Proc. Nat'l. Acad. Sci. USA* 63:458-464). The polymers can be denatured, for example, by heating to about 100°C for 10 minutes, followed by rapid cooling in an ice-salt bath. Binding also can be evaluated in both high (about 0.51M sodium ion) and low (about 0.01M sodium ion) salt.

Binding to deoxyribopolynucleotides, such as poly dAT or poly dA:dC, both double-stranded and single-stranded, also can be evaluated. The four standard homodeoxyribonucleotides also can be tested. Binding to denatured DNA or to disordered deoxyribopolymers can indicate a requirement for a less-ordered structure of the polymer or for a more hydrophobic environment: both may be true. It also can indicate a requirement for binding sites involving reactive groups on the bases that are not available in double-stranded structures. Preferential affinity for any of the four homodeoxyribopolynucleotides, taken together with any binding observed with denatured poly dAT or poly dA:dC, suggests a base specific requirement for binding which can indicate whether potential target organisms will be affected by the agent, depending upon any preferential base usage by the organism.

Binding to various types of ribonucleotides also can be tested, including ribosomal RNA (rRNA), viral RNA (for example from tobacco mosaic virus (TMV), and yeast soluble RNA (sRNA). Binding generally is carried out prior to introduction to the assay device in an aqueous solution at moderate salt concentrations. If binding to DNA is observed only with single-stranded DNA, synthetic ribocopolymers can be used, particularly poly UC and poly AC, because any base pairing in these copolymers would be non-ideal and hence minimal and would enhance the ability to detect any binding to the ribocopolymers. The role of hydrophobic forces in the association of a test compound with a polynucleotide can be evaluated by varying the solvent used for binding. *See*, for example, Seiger (1992) *Advances Protein Chem.* 17:1-68 and Sage and Seiger (1992) *Biochemistry* (Wash) 1:305-317).

Depending upon the potential intended use of the test agent, other macromolecules are also of interest as effectors, including proteins such as receptor sites, substrates for particular

enzymes, structural proteins such as fibrinogen, fibrin, thrombin, albumin, collagen, tubulin, elastin, etc; antigens; antibodies; viral core and coat proteins; extracellular matrix components such as hyaluronic acid, heparin, and synovial fluids; cellular membranes and lipids, such as cholesterol, phosphatidyl choline, brain lipids, nucleic acids; and carbohydrates, such as heparin and the like. Where the binding is specific, the amount of the effector generally is less than the amount of test agents by at least one order of magnitude, usually two orders of magnitude, and in the range of about 10^{-9} M to 10^{-6} M. Subcellular affinity can be evaluated by analyzing differential binding to subcellular organelles, such as cell membranes or other cellular components, cells and tissues or tissue homogenates. The effector used can be prepared by standard methods for preparation of tissue homogenates and/or subcellular fractions from biological sources. A defined concentration on a weight per volume or total protein/volume basis is used. Selective tissue affinities also can be evaluated, for example normal vs tumor tissue and affinity for particular blood components; cellular affinities/affinity to various cell types and/or tissues.

An array of effectors for evaluating the potential bioavailability of a test agent include compositions that mimic one or more biological system which affects the bioavailability of a test agent, for example by degradation or by sequestering of the test agent. Sequestering of the test agent includes binding to the test agent, to form a complex which is not available biologically and/or is eliminated from the body, or to transport the complex into a tissue other than the intended biological target, e.g. adipose tissue instead of brain tissue. Route of administration modeling can be performed by using components integral to a particular route as the effectors.

Examples of such bioavailability effectors include those present at the site of intended introduction into a biological arena of a test agent. For example, where the biological arena is a mammalian subject, the sites of introduction can include lungs, oral, intravenous, intrathecal, rectal, vaginal, intramuscular, subcutaneous, and ocular. The bioavailability following introduction at these sites can be evaluated by exposure of the agent to effectors that are biological fluids such as saliva, using samples and/or extracts of whole blood, serum, plasma, lymph, gastric fluids, synovial fluids, tears, bile, pancreatic juice, feces, and various tissues and extracts such as organ homogenates and/or supernatants from brain, glandular tissue, visceral organs (such as liver), skin, muscle, and gastrointestinal tract (e.g. stomach and intestines) and adipose tissue or proteins, particularly proteolytic enzymes, or proteins known to bind to drugs,

such as serum albumin, from the site of introduction. Conveniently, surrogate systems are available commercially. Surrogate systems that can be employed include vinegar; olive oil; hydrogen peroxide (3%); soluble fiber; starch; and egg/milk protein. These materials represent absorbents in the gastrointestinal tract or agents that my degrade a test compound if it is administered orally.

A variety of effectors can be used to specifically evaluate whether a drug candidate would be efficacious when formulated for administration by a particular route. As an example, for the oral route, differential interaction with and/or affinity for components of saliva, gastric enzymes, acid conditions, gastrointestinal tract, and proteolytic enzymes can be evaluated. In addition, ingested food stuffs can alter absorption and ultimately bioavailability following oral drug administration and can be evaluated by using as effectors foodstuffs such as carbohydrates, fiber, fats, oils and protein. For the intravenous route, affinity for blood components and interaction with liver derived fractions, such as a liver homogenate or liver enzymes, preferably purified enzymes, is evaluated using these substances as effectors. Drugs administered intravenously are often dramatically affected by binding to serum albumin which can be evaluated as an effector. Intrarterial administration of drugs is affected by liver enzymes and subcutaneous drug administration is affected by adipose tissue which can sequester the drug; liver and adipose components can be used as appropriate as effectors. For the intramuscular route, affinity for muscle tissue and components thereof can be evaluated using appropriate samples and/or extracts as effectors.

Where the biological arena is an agricultural setting, bioavailability can be evaluated, depending upon the site of application, by exposure to soil organisms for application to the rhizosphere, or to organisms which colonize a plant part such as leaves, fruit or flowers, for application to a plant part. As appropriate, this evaluation can be combined with an evaluation of the effects of temperature and/or light vs. time with or without stressors such as metal ions or altered salinity on bioavailability to determine persistence, potential breakdown products, and the like. Differential analysis of a compound's affinity for particular plant tissues and soil components also can be assessed.

Toxicological modelling studies also can be performed by evaluating the differential binding of a test agent as an example to DNA, reproductive tissue, brain, and liver. The effect of binding to any of these tissues by an agent also can be used to evaluate e.g. metabolic conversion

by the liver of an agent to a metabolite(s), particularly an active metabolite(s). Moreover, interaction with pharmaceutical excipients can be profiled with this system

An array of effectors for evaluating physical stability as a means of assessing, for example, shelf life of an agent under various conditions of temperature and/or light vs. time can include temperatures from about 4°C to 50°C and are tested by positioning the test apparatus over a controlled heating or cooling means, such as contact heating or cooling, for example electrical; convection heating; forced hot air circulation; immersion of at least a portion of the apparatus in a liquid, for example water of the appropriate temperature, or by incorporating a heating or cooling means into the test apparatus. Likewise, the effect of light can be assessed by exposing the apparatus to light of a wavelength in the infrared through ultraviolet, generally in the visible range, by mounting a light source of the appropriate wavelength over the test apparatus. The light can be constant or can be provided as light cycles. The effect of both temperature and light together also can be assessed.

Following contact of the test agent and the effector for a sufficient time for any interaction to occur, which can be seconds to days, depending on the assay profile model used, flow of solvent is induced. The method used can involve any of a number of separation techniques, including capillary flow electrophoresis, solvent partitioning and/or conventional chromatography, including liquid column chromatography, thin layer chromatography, high performance liquid chromatography, affinity chromatography, or ion exchange chromatography. These systems can be exploited in miniature and/or automated.

Generally the solvent is an aqueous solvent, although other solvents such as acetonitrile can be used depending upon the nature of test agent and the effectors and/or the nature of the interaction under evaluation, such as specific binding, hydrophobic interactions and the like. The solvent used should be one which is able to move the test agent. As an example, where tissue homogenates, or viable cells are used, a culture medium with or without serum or a balanced salt solution can be used so as to maintain viability of the cells. Unless it is an effector, the pH of an aqueous solvent is usually about 4 to 10, preferably, about 5 to 9, more preferably, 6 to 8. The pH is chosen to maintain a significant amount of binding between a test agent and an effector and/or chemical interaction between a test agent and an effector as well as optimal signal generation by a signal producing system used to detect any flow of the test agent through the racetracks. Various buffers can be used to achieve the desired pH and maintain the pH during

the method. Illustrative buffers include borate, acetate, phosphate, carbonate, tris, barbital and the like. The particular buffer employed is not critical, but in individual analyses, one buffer may be preferred over another. Choice of solvents, pH and buffers is within the level of skill of one skilled in the art.

Following contact, if there is no interaction between the test agent and the effector, the test agent flows unimpeded along its racetrack. If there is binding of some or all of the test agent to the effector, flow of the test agent is impeded. If the effector alters the chemical characteristics of the test agent, there is loss of the test agent and formation of at least one new compound, including a conjugate between the test agent and the effector, or a degradation product of the test agent.

Methods of detecting any interactron(s) between an effector and a test agent include visual inspection, densitometry, two-dimensional imaging and video and other methods known to those of skill in the art. The methods of detection can be performed using fluorescence, UV light, visible range or another wavelength. Alternatively, a signal producing system can be used. In one approach, the test agent is labeled and the signal produced from the labeled ligand bound to the effector or the signal produced by the labeled ligand that is chemically or physically sequestered is detected. The label can be any detectable label, including a radionuclide, a fluorescer, or any other detectable label known to those of skill in the art. When it is desired to determine the amount of test agent that is bound to or sequestered by the effector, the amount of signal detected in the presence of the effector is compared to the amount of signal produced in the absence of the effector.

Data concerning each test agent can be analyzed in a variety of ways for developing a profile of a test agent. Qualitatively, test drug/effector interactions can be scored, for example, as unaffected, sequestered, bound or degraded or whatever other interaction is appropriate for the effector and / or test agent under evaluation. Quantitatively, percent of test control compound that is bound or degraded, etc. can be determined either from a single test concentration or in a dose-response type of testing system. Binding constants can be derived from such tests and analyses. Furthermore, metabolites or degradation products derived during the testing can be separated or extracted and chemically analyzed by techniques such as mass spectroscopy and NMR, or other methods known to those of skill in the art. These data are combined and used to create a database of profiles of chemical compounds.

Once the database of profiles has been developed it can be used, as an example, for searching for compounds which have a particular profile by using a desirable set of profile parameters to find a chemical candidate that matches the set of desirable parameters. A list of candidates (first 10 or first 100 or other number) starting with the candidates that have a particular percentage match or better with the desirable set of parameters is then generated. The % match can be quantified as follows:

$$\% \text{ Match} = \frac{\text{Number of Matched Parameters}}{\text{Total Number of Parameters}} \times 100$$

A particular minimum percent match can be specified as a cut-off and in addition some parameters that are "must have" can be specified so that any candidate chemical which lacks these "must have" parameters is omitted from the list, regardless of percent match. As an example, if a chemical is desired with a 50% or better match and the desirable set of profile parameters are nonbinding (B), sequestering (S) or degradating (D) with effectors phosphatidyl choline (P), bovine serum albumin (B), DNA (D), and potassium permanganate (K), this information is entered as a search profile and a search conducted for all compounds with a 50% or better match. All compounds in the database are searched, and those that have a percent match of 50% or greater are printed out as shown in Table 2, below.

Table 2

Printout of Search Results

← Parameters →													Percent Match	Compound
Binding				Sequestering				Degradation						
P	B	D	K	P	B	D	K	P	B	D	K		
-	-	-	-	-	-	-	-	-	-	-	-	100%	A
+	-	-	-	-	-	-	-	-	-	-	-	92%	B
-	-	-	+	+	-	-	-	+	-	-	-	75%	C
+	-	-	+	+	-	-	+	+	+	-	-	50%	D

In the hypothethical search above, four compounds, A, B, C, and D are identified. If the search is further refined and no binding to phosphatidyl choline is identified as a "must have", then only compounds A and C would be identified as candidate chemicals.

The following examples are presented by way of illustration, not of limitation.

EXAMPLES

Materials and Methods:

Test drug / chemical substances:

Ethidium Bromide (EtBr) Sigma E-1385 (5ml) Lot 45H 6750

5-Fluorouracil (5-FU) (Sigma F-6627 (1g) Lot 55H05201)

Doxycycline HCl (Doxy) (Sigma D-9891 (1g) Lot 26H0213)

Epinephrine bitartrate (Epi) (Sigma) (~ 1-5mg/ml in H_20)

Actinomycin D (ActD) (Sigma)

Mitomycin C (MitC) (Sigma)

Effectors:

Oxidizers: Potassium permangenate (KMnO4)(~ 1-5mg/ml in H20); 3% hydrogen peroxide

Blood Protein: Bovine Serum Albumin (BSA) (Fraction V) Sigma A-2153 (5g) Lot 36H1183

Cell Membrane Component: L-α Phosphatidylcholine (PC) Sigma P-5394 Lot 85H 7185

Cell Nucleus Components: Deoxyribonucleic acid (DNA) Sigma (1-5 mg/ml in H20); Ribonucleic acid (RNA); Guanine-Cytosine (G-C) Nucleic acid polymer) (Sigma); Adenine-Thymine (A-T) Nucleic acid polymer (Sigma)

Tissue Homogenates (in Buffer): mouse stomach; liver; intestine; gastrointestinal fluids/substances; saliva, urine, feces, acidic solution (vinegar).

Food Stuffs: Soluble fiber; milk/egg protein; starch; olive oil; mayonnaise

Solvents:

Ethyl Acetate (EtOAc) Sigma 27,052-0 Lot 02235AQ

Acetonitrile Sigma 27-071-7 Lot 053 50TN

Miscellaneous:

TLC plates: silica gel + fluorescent marker Fisher 5x20 cm

Testing Techniques Used In The Examples

Technique A

A 2-5 µl spot of a test drug was placed on 5 x 20 cm TLC plate at origin (~ 5 mm from plate edge). A ~ 5-10 µl spot of an effector was placed 1-2 cm from the test drug spot in the direction of solvent flow. After the spots dried, the plate was developed in a solvent system such that the test drug would come in contact with the effector spot. The plate was developed to 5-10 cm from the origin. The plate was inspected visually by visible or short or longwave UV light.

Technique B

A 1:1 mixture of test compound with an effector was made in a 1.5 ml Eppendorf centrifuge tube. As a function of time, a 2-5 µl sample of the combined mixture was spotted on a TLC plate at the origin. The spot was air dried and then developed in a TLC tank with a solvent system. The solvent front rose from 5-15 cm. After development, the excess solvent was dried in air from the TLC plate. The plate was inspected visually by visible light or short or longwave UV light.

The majority of the initial studies utilized Technique B for efficiency and simplicity.

Example 1

Profile of bioavailability of ethidium bromide, doxycycline and 5-fluorouracil with biomolecule effectors

The purpose of this experiment was to determine whether the interaction of drug compounds with known interactions with particular biomolecules could be detected by thin layer types of chromatographic techniques. The three drug compounds tested were ethidium bromide, doxycycline, and 5-flurouracil. The results are shown in Table 3.

EtBr presents as a liquid; doxy~1-5 mg was dissolved in ethyl acetate (EtOAc)/ ml; 5-FU was dissolved ~1-5 mg/ml in EtOAc; BSA ~1-5 mg/ml was dissolved in distilled water; PC ~1-5 mg/ml was dissolved in EtOAc.

Table 3

Interaction of 5-FU, Doxy or EtBr with PC or BSA

Plate #	Test Agent	Effector	Solvent System	Technique	Result[1]

[1] Movement is relative to the origin.

Plate #	Test Agent	Effector	Solvent System	Technique	Result[1]
1	Doxy 5-FU EtBr none PC or BSA at origin	PC BSA at origin	EtOAc	A	PC alone separated along solvent front; complete movement of 5-FU; others remained at origin.
2	Same as 1	none	Acetonitrile	A	PC alone separated along solvent front; complete movement of 5-FU; others remained at origin.
3	Same as 1	none	Acetonitrile:H_2O 1:1	A	separation of PC along with solvent front; movement of EtBr and 5-FU; dragging from origin of Doxy; BSA remained at the origin.
4	Same as 1	PC Barrier	Acetonitrile:H_2O 1:1	A	same results as 3 appeared to partially sequester in the effector
5	Same as 1	BSA Barrier	Acetonitrile:H_2O 1:1	A	same results as 3 appeared to partially sequester in the effector
6	EtBr 1/100 EtBr 1/10 Doxy 1/100 Doxy 1/10 5-FU 1/00 5-FU 1/10	BSA Barrier	Acetonitrile:H_2O 1:1	A	same results as 3 appeared to partially sequester in the effector
7	Same as #6	PC Barrier	Acetonitrile:H_2O 1:1	A	same results as 3 appeared to partially sequester in the effector

There was an interaction between doxy and both PC and BSA; since neither BSA alone nor doxy alone moves, apparently a conjugate with different properties is formed. Likewise, a conjugate appears to form with PC (although PC does move). There was no interaction between either 5FU or EtBr and either PC or BSA.

Table 4

Interaction of Drug Molecules with Biomolecules and Oxidants

Plate #	Test Agent	Effector	Solvent System	Technique	Result[2]
5 FU 9-4-96	5 FU	PC	Acetonitrile:$H_2$0 3:1	B	No effect
		BSA			Sequester
		DNA			Sequeseter
		$KMN0_4$			No effect
Doxy 9-4-96	Doxycyline	PC	Acetonitrile:$H_2$0 3:1	B	degradant; alteration of absorbence pattern
		BSA			No effect
		DNA			No effect
		$KMN0_4$			Degradant
EthBr 9-4-96	Ethidium Br.	PC	Acetonitrile:$H_2$0 3:1	B	Alteration of migration loss of signal
		BSA			Sequestering
		DNA			Sequestering
		$KMN0_4$			Degradation
Epi 9-4-96	epinephrine	PC	Acetonitrile:$H_2$0 3:1	B	No effect
		BSA			No effect
		DNA			Degradation
		$KMN0_4$			Degradation

Example 2

Evaluation of Drug Interaction with Biomolecules and Oxidants Mixed Together and then Separated by TLC (Thin Layer Chromatography)

The purpose of this experiment was to determine if reactions between test drugs and biomolecules or chemical stressers such as oxidizing agents like potassium permangenate could be detected using small volume reaction mixtures and thin layer chromatography (TLC) to determine the stability of test molecules as well as their affinity for various biomolecules. The test drugs used were Doxy, 5-FU, EtBr, and Epi. In these experiments test solutions of test drugs with biomolecule or $KMnO_4$ were mixed 1:1 (~100µl of each). After mixing, 2-5µl of the mixture were spotted onto 5 x 20 silica cm TLC plates and then separated using Acetonitrile: H_2O (3:1) as described in Example 1 as a solvent system. The mixture was incubated for

approximately 5-10 min. prior to spotting. The results are shown in Table 4, and Figures 1 and 2.

The following general observations were made. 5-FU after a short exposure to test materials had little interaction with PC or $KMN0_4$. However, interaction (streaking) with BSA and DNA (UV, SW) did occur. Doxycycline (UV, LW) had no significant interaction with BSA or DNA. However, alteration of its chromatographic pattern due to affinity or interaction with PC did occur. Also, there was an interaction with or degradation by $KMnO_4$. For Ethidium Bromide, (UV, LW) an interaction or alteration of the chromatogram relative to control EtBr alone with PC BSA DNA and $KMnO_4$ was observed. An aparent reaction with or new degradation product with DNA and $KMnO_4$ occurred with epinephrine (UV, SW). However, there was no apparent interaction with PC and BSA.

5-FU after short exposure to test materials had little interaction with PC or $KMnO_4$ however interaction (streaking) with BSA and DNA (UV,SW 9-04-96) did occur. Doxycycline (plate 9-04-96 UV,LW) had no significant interaction with BSA or DNA. However, alteration of its chromatographic pattern due to affinity or interaction with PC did occur. Also, an interaction or degradation by $KMnO_4$ resulted. for Ethidium Bromide, (9-04-96 UV,LW) an interaction or alteration of the chromatogram relative to control EtBr alone with PC BSA DNA and $KMnO_4$ was identified. An apparent reaction with or new degradation product from DNA and $KMnO_4$ occurred with epinephrine (9-04-96 UV,SW). However no apparent interaction with PC and BSA was observed.

Using the methods described. degradation, sequestering and binding of test agents with effectors were quickly identified. In addition, unexpected interactions were observed between 5-FU and both BSA and DNA, and between doxycycline and PC. Also unexpected was the observed epinephrine degradation by DNA.

Example 3

Interaction of Test Substances with Nucleic Acid Polymers

The purpose of this study was to determine whether binding between test drug substances. nucleic acids or nucleotide copolymers can be detected using small reaction mixtures and different time exposures for their interaction by TLC. The test drugs used were actinomycin D, mitomycin C, and EtBr.

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[2] Movement is relative to the origin.

The following stock solutions were prepared:

250 µl H_2O added to 5 units A-T

100 µl H_2O added to 5 units G-C

500 µl H_2O added to 2 mg ActD

500 µl H_2O added to 2 mg MitoC

EtBr 500 µg/ml stock solution

~ 2 mg of DNA and RNA added to 0.5 ml H_2O

Test mixtures were made 1:1 into Eppendorf tubes, then mixed and the samples incubated at room temperature for ~20-30 minutes or ~24 hours. After incubation, 5 µl samples were spotted onto silica TLC plates, the spots were air dried and developed in a solvent system (Acetonitrile:H_2O (3:1) in a TLC developing tank ~2-5 minutes). The plates were air dried (origin and solvent fronts were first marked). The plates were examined under UV light, either short wave (SW) or long wave (LW). The results are shown in Table 5 below.

Using the techniques and methods described we demonstrated that Actinomycin fragmented or damaged both RNA and G-C polymers. Similarly, mitomycin C altered / fragmented RNA and G-C polymers, but not DNA or A-T polymers. Ethidium bromide was sequestered preferentially by G-C, but polymers as compared to A-T polymers and was completely bound by DNA but not by RNA.

Table 5

Interaction of Actinomycin D, Mitomycin C, and Ethidium bromide with Biopolymers

Plate #	Test Agent	Effector	Solvent System	Technique	Result[3]
1	ActD	DNA	Acetonitrile: H_2O	B	binds
	ActD	RNA	3:1	B	binds
	ActD	A-T		B	minimal binding
	ActD	A-C		B	some binding
	ActD	--		B	moves with solvent front
2	ActD	DNA	Acetonitrile: H_2O	B	some binding
	ActD	RNA	3:1	B	less binding
	ActD	A-T		B	little binding
	ActD	A-C		B	less binding
	ActD	--		B	moves with solvent front
3	MitoC	DNA	Acetonitrile: H_2O	B	some binding
	MitoC	RNA	3:1	B	less binding
	MitoC	A-T		B	little binding
	MitoC	A-C		B	less binding
	MitoC	--		B	moves with solvent front

[3] Movement is relative to the origin.

Plate #	Test Agent	Effector	Solvent System	Technique	Result[3]
4	EtBr	DNA	Acetonitrile: H_20	B	some binding
	EtBr	RNA	3:1	B	less binding
	EtBr	A-T		B	less binding
	EtBr	A-C		B	less binding
	EtBr	--		B	moves close to solvent front
5	MitoC	DNA	Acetonitrile: H_20	B	binds
	MitoC	RNA	3:1	B	some binding
	MitoC	A-T		B	less binding
	MitoC	A-C		B	some binding
	MitoC	--		B	moves with solvent front
6	MitoC	DNA	Acetonitrile: H_20	B	binds
	MitoC	RNA	3:1	B	conjugate formed
	MitoC	A-T		B	minimal binding
	MitoC	A-C		B	minimal binding
	MitoC	--		B	moves with solvent front
7	EtBr	DNA	Acetonitrile: H_20	B	binds
	EtBr	RNA	3:1		no binding
	EtBr	A-T		B	almost no binding
	EtBr	A-C		B	no binding
	EtBr	--		B	moves close to solvent front
8	ActD	DNA	Acetonitrile: H_20	B	binds
	ActD	RNA	3:1	B	some binding
	ActD	A-T		B	little binding
	ActD	A-C		B	some binding
	ActD	--		B	moves with solvent front

Example 4

Evaluation of Oral Bioavailability

The purpose of this study was to determine whether binding between test drug substances and substances/effectors that may affect oral drug delivery could be detected using small reaction mixtures and separation by TLC. The effectors used included biological fluids (BF) such as saliva, feces, urine, foodstuffs (F) such as carbohydrates, proteins, oils, fats, etc. or tissues, homogenates/enzymes (TH) found in the GI tract or metabolic organs such as liver. The test drugs used were Vancomycin (Vanco), Doxycycline (Doxy) and Epinephrine (epi).

For food stuffs a thick solution was prepared (~80 mg/ml in water) while biological fluids were used as acquired. Tissue homogenates were generated from mouse tissues diluted ~1:1 with phosphate buffered saline and frozen prior to use in the experiment. Test mixtures were made 1:1 into Eppendorf tubes then mixed and the samples incubated at room temperature for ~20-30 minutes or ~24 hours. After incubation,

~5 µl samples were spotted onto silica TLC plates, the spots were air dried and the plates developed in a solvent system (acetronitrile: H_20 (3:1) in a TLC developing tank ~2-5 minutes. The plates were air dried (origin and solvent fronts were first marked). The plates were examined

under UV light, either short wave (SW) or long wave (LW). The results are shown in Table 7. The results show that epinephrine, which is not administered orally, would be eliminated from further consideration for oral administration because it is degraded by homogenates from stomach and intestinal tissue. Moreover, vancomycin, which is a drug given orally but which is not absorbed into the blood stream, is degraded by food stuffs (soluble fiber) and stomach tissue homogenates and would be eliminated in favor of other drugs with a better profile. On the other hand, doxycycline, which is 100% bioavailable after oral administration, is not degraded or bound by any of the test substances studied although it is sequestered by some food stuffs and substances in the stomach homogenate. This drug then would be retained as a possible drug candidate for oral administration for further development.

Table 7

Evaluation of Epinephrine, Doxycycline, and Vancomycin for Oral Availability

Plate #	Test Agent	Effector	Solvent System	Result
1	saliva	Epinephrine	Acetonitrile/H_2O 3:1	no effect
2	vinegar	Epinephrine	Acetonitrile/H_2O 3:1	no effect
3	urine	Epinephrine	Acetonitrile/H_2O 3:1	no effect
4	feces	Epinephrine	Acetonitrile/H_2O 3:1	sequestration
5	H_2O_2 (3%)	Epinephrine	Acetonitrile/H_2O 3:1	alteration of spot movement
6	olive oil	Epinephrine	Acetonitrile/H_2O 3:1	degradation/ sequestration
7	mayonnaise	Epinephrine	Acetonitrile/H_2O 3:1	binding
8	soluble fiber	Epinephrine	Acetonitrile/H_2O 3:1	no effect
9	starch	Epinephrine	Acetonitrile/H_2O 3:1	no effect
10	egg/milk protein	Epinephrine	Acetonitrile/H_2O 3:1	no effect
11	stomach homogenate	Epinephrine	Acetonitrile/H_2O 3:1	
12	intestine homogenate	Epinephrine	Acetonitrile/H_2O 3:1	
13	liver homogenate	Epinephrine	Acetonitrile/H_2O 3:1	
14	liver supernatant	Epinephrine	Acetonitrile/H_2O 3:1	
15	drug alone control	Epinephrine	Acetonitrile/H_2O 3:1	
1	saliva	Doxycycline	Acetonitrile/ H_2O 3:1	change in absorbence SW/LW
2	vinegar	Doxycycline	Acetonitrile/ H_2O 3:1	no effect
3	urine	Doxycycline	Acetonitrile/ H_2O 3:1	binding/ degradation
4	feces	Doxycycline	Acetonitrile/ H_2O 3:1	binding
5	H_2O_2 (3%)	Doxycycline	Acetonitrile/ H_2O 3:1	no effect
6	olive oil	Doxycycline	Acetonitrile/ H_2O 3:1	binding
7	mayonnaise (Pecnutoulter)	Doxycycline	Acetonitrile/ H_2O 3:1	binding
8	soluble fiber	Doxycycline	Acetonitrile/ H_2O 3:1	sequestration
9	starch	Doxycycline	Acetonitrile/ H_2O 3:1	sequestration
10	egg/milk protein	Doxycycline	Acetonitrile/ H_2O 3:1	binding

11	stomach homogenate	Doxycycline	Acetonitrile/ H_2O 3:1	degradation[1]
12	intestine homogenate	Doxycycline	Acetonitrile/ H_2O 3:11	sequestration
13	liver homogenate	Doxycycline	Acetonitrile/ H_2O 3:1	sequestration degradation
14	liver supernatant	Doxycycline	Acetonitrile/ H_2O 3:1	seqiestration degradation
15	drug control	Doxycycline	Acetonitrile/ H_2O 3:1	
1	saliva	Vancomycin	Acetonitrile/ H_2O 3:1	change in absorbence
2	vinegar	Vancomycin	Acetonitrile/ H_2O 3:1	no effect
3	urine	Vancomycin	Acetonitrile/ H_2O 3:1	no effect
4	feces	Vancomycin	Acetonitrile/ H_2O 3:1	binding
5	H_2O_2 (3%)	Vancomycin	Acetonitrile/ H_2O 3:1	no effect
6	olive oil	Vancomycin	Acetonitrile/H_2O 3:1	binding/change in absorbence
7	mayonnaise	Vamcomycin	Acetonitrile/H_2O 3:1	binding/change in absorbence
8	soluble fiber	Vamcomycin	Acetonitrile/H_2O 3:1	binding/change in absorbence
9	starch	Vamcomycin	Acetonitrile/H_2O 3:1	no effect/binding?
10	egg/milk protein	Vamcomycin	Acetonitrile/H_2O 3:1	binding/change in absorbence
11	stomach homogenate	Vamcomycin	Acetonitrile/H_2O 3:1	degradation/change in absorbence
12	intestine homogenate	Vamcomycin	Acetonitrile/H_2O 3:1	degradation/change in absorbence
13	liver homogenate	Vamcomycin	Acetonitrile/H_2O 3:1	degradation/change in absorbence
14	liver supernatant	Vamcomycin	Acetonitrile/H_2O 3:1	degradation/change in absorbence
15	drug alone control	Vamcomycin	Acetonitrile/H_2O 3:1	degradation/change in absorbence

Example 5

Development of a Chemical Profile

Tables 9-12 summarize the types of interactions identified by the rapid chemical profiling technique in the experiments outlined in Examples 1-4. Using a chemical stress effector, potassium permanganate, degradation of doxycycline, ethidium bromide and epinephrine was demonstrated. (Table 9). This is consistent with the known properties of these compounds relating to sensitivity to oxidation.

Table 9 Examples of Individual Drug/Effector Interactions Observed With the Test Methods Used: Chemical Effectors

Drug	Effector	Reaction/Interaction	Example #
Doxycycline	$KMnO_4$	Degradations	1,2
Ethidium Br	$KMnO_4$	Degradation	1,2
Epinephrine	$KMnO_4$	Degradation	1,2

Using biological macromolecule effectors, including various forms of nucleic acid

[4] change in absorbance LW(UV)

polymers, it was possible to detect different binding, sequestering and degradation patterns summarized in (Table 10) among the compounds tested, which again were consistent with known properties of these compounds. Among these observations, only the binding of Ethidium bromide to DNA was a known property; the remaining interactions constitute new information concerning macromolecular interactions of 5-FU, Doxycycline and Ethidium bromide.

Table 10 Examples of Individual Drug / Effector Interactions Observed with the Test Methods Used: Biological Effectors;

Drug	Effector	Reaction/Interaction	Example #
5-FU	BSA DNA	Sequestered Sequestered	1,2
Doxycycline	PC	Alteration / Sequestered	1,2
Ethidium Br	DNA (ds) PC G-C A-T	Binding of chromatogram Sequester / Binding Sequester	1,2,3 3
Epinephrine	DNA	Degradation	2
Actinomycin D	RNA C-C	Sequestered/ Bound Sequestered/ Bound	3
Mitomycin C	DNA (ds) A-T RNA	Binding Binding Fragmentation	3

Specific details about a compound's attributes and vulnerabilities can quickly be identified using the rapid chemical profiling methodology. For example, using the data in Table 10, investigators can quickly identify two compounds, Mitomycin C and Ethidium bromide, that have greater preferences to bind to DNA than RNA. Conversely, Actinomycin D may have unique opportunities as an inhibitor of transcription and translation perhaps affecting protein synthesis. Moreover, the G-C rich regions of nucleic acid polymers, are vulnerable to strand breakage/fragmentation by Actinomycin D. These observations may offer insights for additional compounds to screen possible mechanisms of action, and / or possible new uses for these

compounds.

Example 6

Evaluation of Profiles to Identify Chemicals with Desired Characteristics

In order to automate the search for chemicals with a desired profile, each of four drugs tested in the examples above, was scored for each effector tested (see Table 11). The following scoring system was applied to describe the test results in the examples provided below:

Table 8

Quantitative Scoring System

Score	Observation
+1	Binding of test molecule retained at origin Degradation of test molecule Sequestering of test molecule altering its chromatogram
0	Ambiguous result
-1	No binding No degradation No sequestering observed

If the desired chemical profile is that of an antimetabolite, such as an anticancer agent, the desired set of characteristics are nonbinding and/or non-sequestering by any effector except DNA ("must have") for a total score of -10. The resulting profiles are shown in Table 11. Of the four drugs shown, three are identified as having the "must have" characteristics of binding and / or sequestering of DNA (D): ethidium bromide, 5-fluorouracil and doxycycline HCl, with percentage matches of 40%, 60% and 40% respectively. If in addition a parameter of greater than 50% match is added, then only one compound remains that would be further evaluated as an antineoplastic agent, 5-fluorouracil, which does show a DNA interaction (sequestering).

Table 11

Profile of Four Compounds / Drugs of Diverse Structure and Biological Activity: Study of Interactions with Biomolecules and a Chemical Oxidizing Agent

Test Drug 5 FU	Scoring Review

	Binding P* B D K	Sequestering P B D K	Degradation P B D K	Score Total
"expected" results	-1 -1 -1 -1	-1 -1 -1 -1 0 0	-1 -1 -1 -1 0	-9 -12
actual results	-1 -1 -1 -1	-1 +1 +1 -1	-1 0 0 -1	-6

Test Drug Ethidium Br. Scoring Review

	Binding P B D K	Sequestering P B D K	Degradation P B D K	Score Total
"expected" results	-1 0 +1 -1	0 +1 +1 -1	-1 -1 -1 -1	-4
actual results	-1 0 +1 -1	-1 0 +1 -1	+1 -1 -1 +1	-4

Test Drug Epinephrine HCl Scoring Review

	Binding P B D K	Sequestering P B D K	Degradation P B D K	Score Total
"expected"results	-1 0 -1 -1	0 0 -1 -1 +1	-1 -1 -1 +1	-6→ -8
actual results	-1 -1 -1 -1	-1 0 0 -1	-1 -1 +1 +1	-6

Test Drug Doxycycline HCl Scoring Review

	Binding P B D K	Sequestering P B D K	Degradation P B D K	Score Total
"expected" results	-1 +1 - 1 -1	+1 +1 -1 -1	-1 -1 -1 0	-5
actual results	-1 -1 0 0	+1 -1 +1 -1	0 -1 -1 +1	-4

*P= Phosphotidyl Choline; B= Bovine Serum Albumin; D= DNA; and K= Potassium Permanganate

TABLE 12 Test Compounds Studied in Experimental Examples
Known Properties and Properties Identified by RDP

Drug/ Compound	Class	Chemical Family	MW	Sol.	Route of Admin.	Dose/ Schedule	Mechanics of Actions	Toxicities	Other Features	Features screened for and identified by RDP	References
5-Fluorouracil	antineoplastic/ antimetabolite	fluorinated pyrimidine	130	sparingly in water	i.v.	12 mg/kg D 1-4	inhibits DNA synthesis lesser extent. RNA synthesis S-phase	leukopenia, GI toxicity	distributes through the body including blood brain barrier	no binding to biomolecules (DNA, membrane lipids, serum albumin- no degradation by oxidizing agent	Pharmacia package insert Aducil 4/01/96
Epinephrine HCl	sympathomimetic hormone	substituted benzenediol	183	sparingly sol in water	i.m.s.c.	0.2 - 1.0 mg	vasoconstriction	cardiac arrhythmiapulmonary edema	readily destroyed by alkalies and oxidizing agents including oxygen, chlorine bromine, iodine, permansenates chromates nitrites & salts of easily reducible metals especially iron	sensitivity to being degraded (oxidizing agent and DNA?)	PDR generics 2nd ed. 1996 p. 1129
Doxycycline HCl	antibiotic	oxytetracyclin	462	sol in water	p.o.	100 mg/12 hr.	inhibits protein synthesis	GI skin blood (hemolytic anemia)	readily absorbed ~100% bound to plasma proteins to varying degrees low affinity to calcium binding highly stable in normal human serum conc. by liver in the bile excreted in urine and feces in active form	sequestering by serum albumin and discovered sequestering by lipid (phosphetidyl choline) degraded by oxidizing agents	PDR 1999 p. 2427
Ethidium Bromide	tryponosome antiprotozoal (vet) DNA & RNA binding intercalcating agent: frameshift mutagen	subs phenanthrium	394	sol in H_2O	unk.	unk.	nucleic acid intercalation; inhibit DNA polymerase		frameshift mutagen	binding to DNA degraded by oxidizing agents	Merck 12 809 (homidium)

Drug/ Compound	Class	Chemical Family	MW	Sol.	Route of Admin	Dose/ Schedule	Mechanics of Actions	Toxicities	Other Features	Features screened for and identified by RDP	References
Actinomycin D	Antibacterial antineoplastic	chromopeptide	1255	in water	i.v.	every two weeks	Interacts directly with DNA / Lesser extent with RNA	cardiotoxicity?	50-90% binding to nuclei High levels in kidney, liver & spleen	Binding and fragmentation of RNA and G-C polymers	Foye p272
Mitomycin C	antitumor antibiotic	subst. methyl azirinol pyrrolor indolo dione	334	in water	i.v.	20mg/m² / 6-8 weeks	inhibit DNA synthesis G + C content correlates with the degree of cross linking at high concentration cellular RNA + protein systhesis creates suppression	carcinogen (mice + rats) marrow suppressive	rapidly cleared i.v. T 1/2 =17 min. metabolized by liver and other tissues 10% excreted unchanged in urine	Binding to DNA and A-T polymers Fragmentation of RNA and G-C polymers	PDR 1999 p787 Merck 12th p1063
Vancomycin HCl	antibiotic	tricyclic glycopeptide	1486	in water	oral	250 mg/8hr x 7 doses	inhibit bacteria cell wall synthesis RNA synthesis	nephro, oto hemato toxicities	poorly absorbed after oral administration ≤ 1.0% in blood or urine	Interaction with Foodstuffs and biological Fluids and Tissues Binding to Foodstuffs	PDR 1999 p1683

In comparison to "expected" data as identified in the literature (Table 13) correlative as well as some unexpected observations can be identified for each of the test drugs utilizing the observations for the four compounds of distinct chemical structures (Table 9). In many cases, there were no literature reports identified that discussed interactions such as those evaluated. For example, no reports of 5.FU interactions with albumin or doxycycline interaction with membrane lipids were found in the current literature.

The above results demonstrate the feasibility of using the rapid drug profiling method to obtain accurate information regarding particular characteristics of chemical compounds which can be used to predict how a particular chemical compound will react and function *in vivo*.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporate by reference.

The invention now having been fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for high throughput profiling of characteristics of an agent, said method comprising:

contacting simultaneously on a solid support a parallel array of effectors with multiple replicates of said agent under dynamic conditions and for a time sufficient for a multiplicity of detectable migration patterns to develop as a result of an effect of each of said effectors on said agent as compared to a control, whereby based upon said multiplicity of detectable migration patterns a profile of characteristics of said agent is obtained.

2. The method according to Claim 1, wherein said effectors are chemical effectors.

3. The method according to claim 2, wherein said chemical effectors alter pH, ionic strength or redox of said dynamic conditions.

4. The method according to Claim 1, wherein said effects is degradation of said agent.

5. The method according to Claim 4, wherein said degradation is detected by a change in migration pattern of said agent on said solid support under said dynamic conditions.

6. The method according to Claim 5, wherein detection of said change in migration pattern is by a method selected from the group consisting of ultraviolet inspection, infrared inspection, radioactive scanning, and visual inspection of said solid support.

7. The method according to Claim 6, wherein said detection is automated.

8. The method according to Claim 7, wherein said visual inspection is imaging.

9. The method according to Claim 8, wherein said change in migration pattern is quantified by disitcation of images for computer analysis.

10. The method according to Claim 5, wherein detection of said change in migration pattern is by means of a detectable tag attached to said agent.

11. The method according to Claim 10, wherein said detectable tag is selected from the group consisting of a fluorescent tag, a radionuclide, a chemiluminescent tag, and an antibody.

12. The method according to Claim 5, wherein a percentage of said degradation is determined by comparison of the amount of degraded agent and the amount of unaltered agent.

13. The method according to any one of Claims 6, 10, or 12, wherein said detection is at multiple time intervals.

14. The method according to Claim 4, wherein said effect is alteration of the structure of one or more of said non-target molecules by said agent.

15. The method according to Claim 4, further comprising the step of identifying a product of said degradation of said agent.

16. The method according to Claim 1, where said effectors are biological effectors.

17. The method according to claim 16, wherein said biological effectors are non-target molecules for said agent.

18. The method according to Claim 17 wherein said non-target molecules are selected from the group consisting of DNA, protein, carbohydrate and lipid.

19. The method according to Claim 18, wherein said protein is an enzyme.

20. The method according to Claim 19, wherein said enzyme is a proteolytic enzyme.

21. The method according to Claim 17, wherein said effect is binding of said agent to one or more of said non-target molecules.

22. The method according to Claim 21, wherein said binding is selected from the group consisting of retention, entrapment, and covalent.

23. The method according to Claim 21, wherein said binding is non-covalent binding.

24. The method according to Claim 23, wherein said non-covalent binding is non-specific binding.

25. The method according to Claim 16, wherein said biological effectors are biological materials.

26. The method according to Claim 25, wherein said biological materials are one or more selected from the group consisting of isolated mammalian tissues, biological fluids, cultured mammalian cells, and biopsy specimens.

27. The method according to Claim 26, wherein extracts are prepared of said biological materials.

28. The method according to Claim 26, wherein said biological fluids are selected from the group consisting of blood, cerebrospinal fluid, saliva, gastrointestinal fluid, and a homogenate of a tissue.

29. The method according to Claim 17, wherein said effect is alteration of the structure of one or more non-target molecules by said agent.

30. The method according to Claim 1, wherein said multiple replicates of said agent each contain less than 10 mg of said agent.

31. The method according to Claim 30, wherein said multiple replicates of said agent each contain less than 0.001 mg of said agent.

32. A method for obtaining a multidimensional space of characteristics of an agent, said method comprising:

combining characteristics obtained from multiple profiles of characteristics, wherein each profile is obtained according to the method of Claim 1, whereby a fingerprint of said agent is obtained.

33. The method according to Claim 32, wherein said multiple profiles of characteristics are one or more profiles selected from the group consisting of profiles of physical instabilities, chemical instabilities, and altered bioavailability.

34. The method according to Claim 32, wherein said agent is a pharmaceutical composition.

35. A method for determining chemical stability of a pharmaceutical composition, said method comprising:

contacting simultaneously a combinatorial array of chemicals with said pharmaceutical composition under dynamic conditions and for a time sufficient to determine the effect of one or more of said chemicals on said pharmaceutical composition as compared to one or more control, whereby chemical stability of said pharmaceutical composition is determined.

36. The method according to Claim 35, wherein said chemicals are selected from the group consisting of oxidizing agents, reducing agents, metal ions, acids, and bases.

37. A method for determining physical stability of a pharmaceutical composition, said method comprising:

contacting simultaneously a combinatorial array of physical effectors with said pharmaceutical composition under dynamic conditions and for a time sufficient to determine the effect of one or more of said physical effectors on said pharmaceutical

composition as compared to one or more control, whereby physical stability of said pharmaceutical composition is determined.

38. The method according to Claim 37, wherein said physical effectors are one or more effectors selected from the group consisting of altered temperature of said dynamic conditions, altered light of said dynamic conditions, and magnetic fields.

39. A method for determining a profile of bioavailability of a pharmaceutical composition, said method comprising:

contacting simultaneously a combinatorial array of biological molecules with said pharmaceutical composition under dynamic conditions and for a time sufficient to determine the effect of one or more of said biological molecules on said pharmaceutical composition as compared to one or more control, whereby a profile of bioavailability of said pharmaceutical composition is determined.

40. A method for identifying agents which bind to a target molecule of interest, said method comprising:

contacting simultaneously on a solid support a parallel array of agents with multiple replicates of said target molecule of interest under dynamic conditions and for a time sufficient for a detectable migration patterns to develop as a result of an interaction between said agent and said target molecule of interest as compared to a control, whereby based upon said detectable migration patterns said profile of characteristics of said agent is obtained.

41. The method according to Claim 40, wherein said target molecule of interest comprises a nucleic acid or a protein.

4.2 The method according to Claim 41, wherein said nucleic acid is DNA.

43. The method according to Claim 41, wherein said protein is a cell membrane receptor.

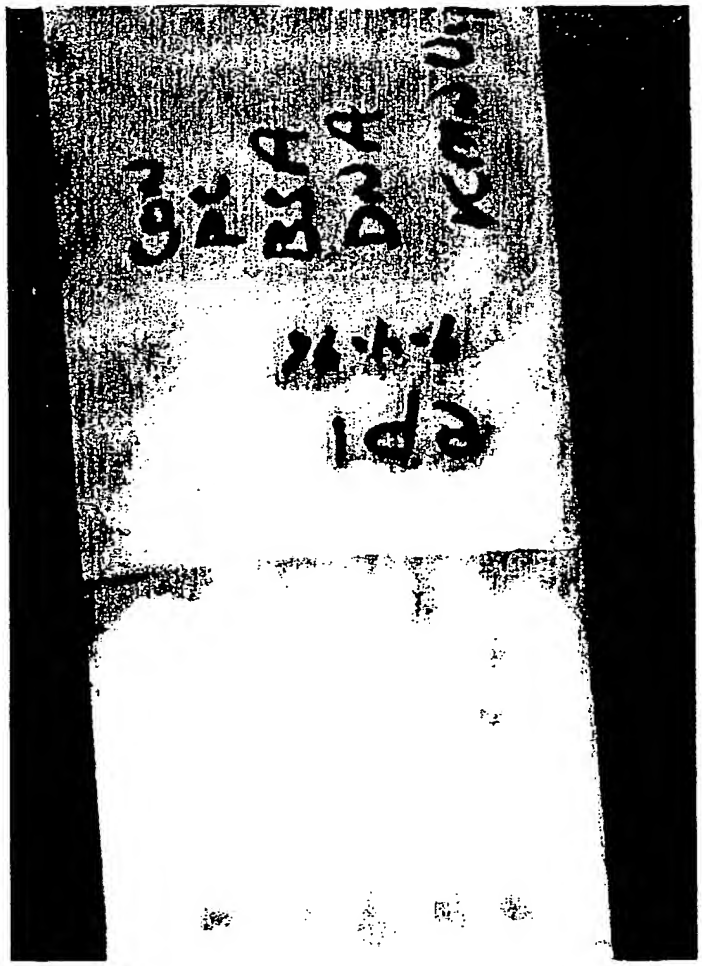

FIG. 1

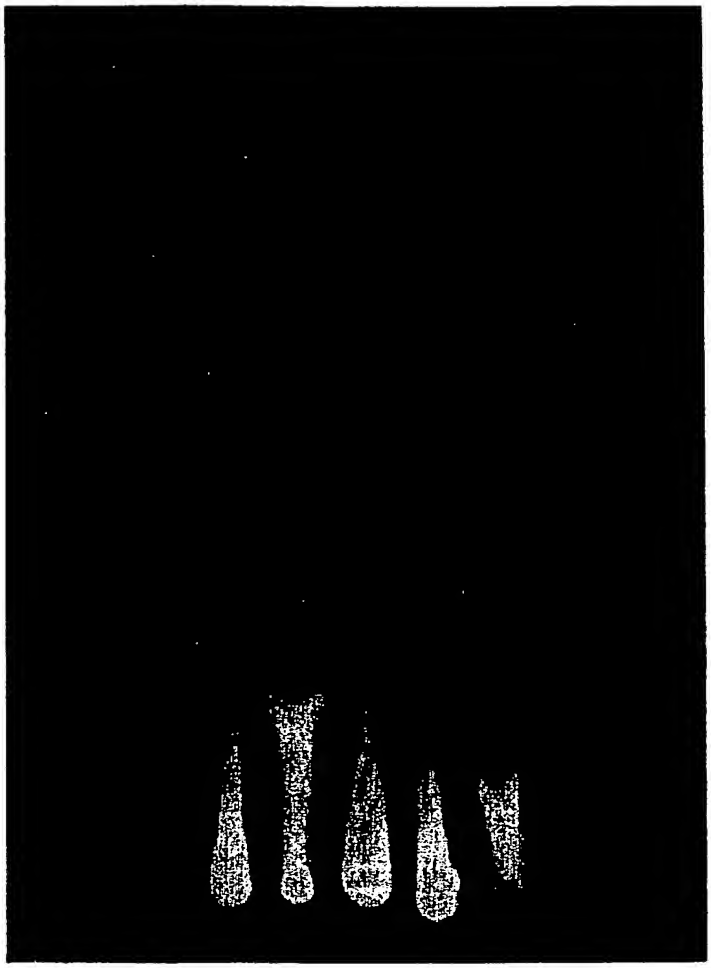

FIG. 2

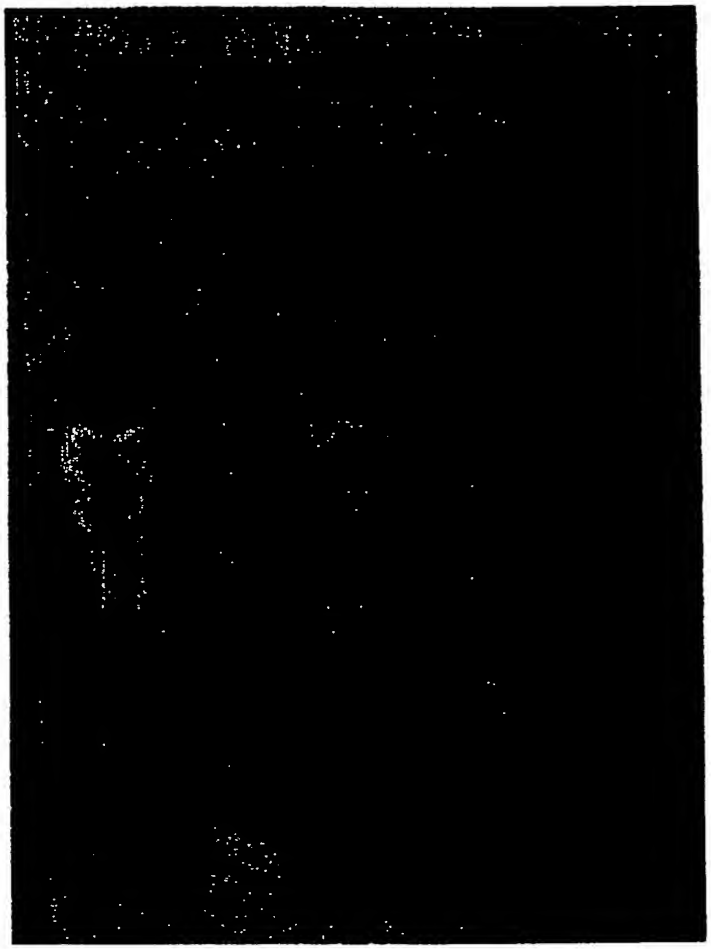

FIG. 3

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(74) Agents: **RAE-VENTER, Barbara** et al.; Rae-Venter Law Group, P.C., P.O. Box 60039, Palo Alto, CA 94306-0039 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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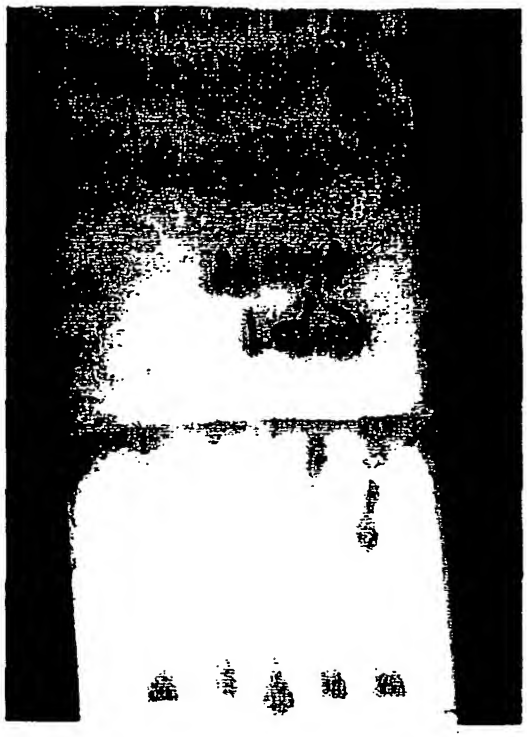
[Continued on next page]

(54) Title: HIGH THROUGHPUT CHEMICAL PROFILING



(57) Abstract: Methods are described for rapid profiling of characteristics of chemical compounds that are useful indicators of the potential efficacy of individual chemical compounds for a particular intended use and/or desired route of administration, as well as information relative to potential chemical and physical stability. The methods use small (<10 mg) amounts of a chemical compound to obtain a complete profile of characteristics. A database generated from the profiles can be searched to identify chemical compounds having a desired set of characteristics.

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patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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INTERNATIONAL SEARCH REPORT

Inten ..nal Application No
PCT/US 00/24093

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12M1/34 G01N33/543 G01N27/447

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
11 December 2000	08.05.01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Zinngrebe, U

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INTERNATIONAL SEARCH REPORT

Inter ɔnal Application No
PCT/US 00/24093

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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4

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 00/24093

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-34,40-43

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-34,40-43

   Detection unimpeded by several substances on the same spot

2. Claims: 35-36

   Selection of effectors to determine chemical stability

3. Claims: 37-38

   Selection of effectors to determine physical stability

4. Claim : 39

   Selection of effectors to determine bioavailability

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte onal Application No
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Information on patent family members

Inte. .onal Application No
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